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<p>(21) International Application Number: PCT/EP00/03730</p> <p>(22) International Filing Date: 26 April 2000 (26.04.00)</p> <p>(30) Priority Data:</p> <table border="0"><tr><td>9909650.5</td><td>27 April 1999 (27.04.99)</td><td>GB</td></tr><tr><td>9916655.5</td><td>15 July 1999 (15.07.99)</td><td>GB</td></tr></table> <p>(71) Applicant (for all designated States except US): SMITHKLINE BEECHAM P.L.C. [GB/GB]; New Horizons Court, Brentford, Middlesex TW8 9EP (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): IRVING, Elaine, Alison [GB/GB]; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB). PARSONS, Andrew, A. [GB/GB]; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB).</p> <p>(74) Agent: BLAKEY, Alison, Jane; SmithKline Beecham Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).</p>		9909650.5	27 April 1999 (27.04.99)	GB	9916655.5	15 July 1999 (15.07.99)	GB	<p>(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>
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<p>(54) Title: NOVEL TREATMENT</p> <p>(57) Abstract</p> <p>The use of Raf kinase inhibitors in the treatment of neurotraumatic diseases.</p>								

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NOVEL TREATMENT

This invention relates to the use of Raf kinase inhibitors in the treatment of neurotraumatic diseases.

5 Raf protein kinases are key components of signal transduction pathways by which specific extracellular stimuli elicit precise cellular responses in mammalian cells. Activated cell surface receptors activate ras/rap proteins at the inner aspect of the plasmamembrane which in turn recruit and activate Raf proteins. Activated Raf proteins phosphorylate and activate the intracellular protein kinases MEK1 and MEK2. In turn, 10 activated MEKs catalyse phosphorylation and activation of p42/p44 mitogen-activated protein kinase (MAPK). A variety of cytoplasmic and nuclear substrates of activated MAPK are known which directly or indirectly contribute to the cellular response to environmental change. Three distinct genes have been identified in mammals that encode Raf proteins; A-Raf, B-Raf and C-Raf (also known as Raf-1) and isoformic 15 variants that result from differential splicing of mRNA are known.

Inhibitors of Raf kinases have been suggested for use in disruption of tumor cell growth and hence in the treatment of cancers, e.g. histiocytic lymphoma, lung adenocarcinoma, small cell lung cancer and pancreatic and breast carcinoma.

20 It has now been discovered that Raf inhibitors are useful in the treatment and/or prophylaxis of disorders associated with neuronal degeneration resulting from ischemic events, including cerebral ischemia after cardiac arrest, stroke and multi-infarct dementia and also after cerebral ischemic events such as those resulting from surgery and/or during childbirth.

25 Thus according to the invention there is provided a method of treatment or prophylaxis of a neurotraumatic disease, in a mammal in need thereof, which comprises administering to said mammal an effective amount of a Raf inhibitor.

30 According to the invention there is also provided the use of a Raf inhibitor in the manufacture of a medicament for the prophylactic or therapeutic treatment of a disease state in a human, or other mammal, which is exacerbated or caused by a neurotraumatic event.

The Raf inhibitor for use in the invention is preferably a B-Raf inhibitor.

35 Neurotraumatic diseases/events as defined herein include both open or penetrating head trauma, such as caused by surgery, or a closed head trauma injury, such as caused by an injury to the head region. Also included within this definition is ischemic stroke, particularly to the brain area, transient ischemic attacks following coronary by-pass and cognitive decline following other transient ischemic conditions.

40 Ischemic stroke may be defined as a focal neurologic disorder that results from insufficient blood supply to a particular brain area, usually as a consequence of an embolus, thrombi, or local atheromatous closure of the blood vessel. Roles for stress stimuli (such as anoxia), redox injury, excessive neuronal excitatory stimulation and inflammatory cytokines in this area has been emerging and the present invention

provides a means for the potential treatment of these injuries. Relatively little treatment, for an acute injury such as these has been available.

Models of closed head injuries and treatment with mixed 5-LO/CO agents is discussed in Shohami *et al*, *J. Vasc. & Clinical Physiology and Pharmacology*, (1992), 3(2), 99-107.

Suitable Raf inhibitors for use in the method of the invention include small molecule and peptides. The definition Raf inhibitor is also intended to encompass antisense constructs that result in a reduced expression of a Raf kinase within a target cell.

Small molecule Raf inhibitors include those described in GB2306108, WO98/22103, WO98/52559, WO98/50370, WO99/10325 and WO99/17759.

A particular Raf inhibitor that may be mentioned is 4-(4-chloro-3-hydroxyphenyl)-2-phenyl-5-(4-pyridyl)-1H-imidazole. The use of this compound for the treatment of Raf-mediated cancers is described in Poster 3793, American Association of Cancer Research, New Orleans, April 1998.

Antisense Raf inhibitors include those described in WO97/10829, US5656612, WO99/02167, US5872232 and WO96/39415.

In order to use Raf inhibitors in therapy, they will normally be formulated into a pharmaceutical composition in accordance with standard pharmaceutical practice.

Inhibitors may conveniently be administered by any of the routes conventionally used for drug administration, for instance, parenterally, orally, topically or by inhalation. Inhibitors may be administered in conventional dosage forms prepared by combining it with standard pharmaceutical carriers according to conventional procedures. Inhibitors may also be administered in conventional dosages in combination with a known, second therapeutically active compound. These procedures may involve mixing, granulating and compressing or dissolving the ingredients as appropriate to the desired preparation. It will be appreciated that the form and character of the pharmaceutically acceptable carrier is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The pharmaceutical carrier employed may be, for example, either a solid or liquid. Exemplary of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid and the like. Exemplary of liquid carriers are syrup, peanut oil, olive oil, water and the like. Similarly, the carrier or diluent may include time delay material well known to the art, such as glyceryl monostearate or glyceryl distearate alone or with a wax.

A wide variety of pharmaceutical forms can be employed. Thus, if a solid carrier is used, the preparation can be tableted, placed in a hard gelatin capsule in powder or pellet form or in the form of a troche or lozenge. The amount of solid carrier will vary

widely but preferably will be from about 25mg to about 1g. When a liquid carrier is used, the preparation will be in the form of a syrup, emulsion, soft gelatin capsule, sterile injectable liquid such as an ampoule or nonaqueous liquid suspension.

Inhibitors are preferably administered parenterally, that is by intravenous, intramuscular, subcutaneous intranasal, intrarectal, intravaginal or intraperitoneal administration. The intravenous form of parenteral administration is generally preferred. Appropriate dosage forms for such administration may be prepared by conventional techniques.

Inhibitors may also be administered orally. Appropriate dosage forms for such administration may be prepared by conventional techniques.

Inhibitors may also be administered by inhalation, that is by intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as aerosol formulations, may be prepared by conventional techniques.

Inhibitors may also be administered topically, that is by non-systemic administration. This includes the application of the inhibitors externally to the epidermis or the buccal cavity and the instillation of such a compound into the ear, eye and nose, such that the compound does not significantly enter the blood stream.

For all methods of use disclosed herein for inhibitors, the daily oral dosage regimen will preferably be from about 0.1 to about 80 mg/kg of total body weight, preferably from about 0.2 to 30 mg/kg, more preferably from about 0.5 mg to 15mg. The daily parenteral dosage regimen about 0.1 to about 80 mg/kg of total body weight, preferably from about 0.2 to about 30 mg/kg, and more preferably from about 0.5 mg to 15mg/kg. The daily topical dosage regimen will preferably be from 0.1 mg to 150 mg, administered one to four, preferably two or three times daily. The daily inhalation dosage regimen will preferably be from about 0.01 mg/kg to about 1 mg/kg per day. It will also be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of the inhibitors will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular patient being treated, and that such optimums can be determined by conventional techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of doses of the inhibitors given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determination tests.

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

The invention will now be described by reference to the following examples which are merely illustrative and are not to be construed as a limitation of the scope of the present invention.

EXAMPLE

4-(4-Chloro-3-hydroxyphenyl)-2-phenyl-5-(4-pyridyl)-1H-imidazolea) 4-(4-Chloro-3-methoxyphenyl)-2-phenyl-5-(4-pyridyl)-1H-imidazole

The sub-title compound was prepared from 4-chloro-3-methoxybenzoic acid, via its acid chloride, by analogy to the five step procedure (general method B) described by T. F. Gallagher *et al*, *Bioorg. Med. Chem.*, (1997), 5, 49: MS *m/z* 362/364 MH⁺ (electrospray).

b) 4-(4-Chloro-3-hydroxyphenyl)-2-phenyl-5-(4-pyridyl)-1H-imidazole

Boron tribromide (5.6 ml, 1M in dichloromethane, 5.6mmol) was added to an ice-cooled solution of the product of step a) (670 mg, 1.85 mmol) in dichloromethane (20 ml). The solution was allowed to warm to ambient temperature and stirred for a further 16h. 5M hydrochloric acid (5 ml) was then added and the mixture heated to reflux for 30 min. After cooling the solution was made alkaline with 40% aqueous sodium hydroxide and the solid product collected by filtration. The residue was washed with ethyl acetate to afford the title compound as a yellow solid: MS *m/z* 348/350 MH⁺ (electrospray).

BIOLOGICAL EXAMPLES

The activity of compounds as Raf inhibitors may be determined by the following *in vitro* assay:

20 Raf Kinase assay

Activity of human recombinant B-Raf protein was assessed *in vitro* by assay of the incorporation of radiolabelled phosphate to recombinant MAP kinase (MEK), a known physiologic substrate of B-Raf. Catalytically active human recombinant B-Raf protein was obtained by purification from Sf9 insect cells infected with a human B-Raf recombinant baculovirus expression vector. To ensure that all substrate phosphorylation resulted from B-Raf activity, a catalytically inactive form of MEK was utilised. This protein was purified from bacterial cells expression mutant inactive MEK as a fusion protein with glutathione-S-transferase (GST-kdMEK).

30 Method: Standard assay conditions of B-Raf catalytic activity utilised 3ug of GST-kdMEK, 10uM ATP and 2uCi ³³P-ATP, 50mM MOPS, 0.1mM EDTA, 0.1M sucrose, 10mM MgCl₂, plus 0.1% dimethylsulphoxide (containing compound where appropriate) in a total reaction volume of 30ul. Reactions were incubated at 25°C for 90 minutes and reactions terminated by addition of EDTA to a final concentration of 50uM. 10ul of reaction was spotted to P30 phosphocellulose paper and air dried. Following four washes in ice cold 10% trichloroacetic acid, 0.5% phosphoric acid, papers were air dried prior to addition of liquid scintillant and measurement of radioactivity in a scintillation counter.

40 Results: 4-(4-Chloro-3-hydroxyphenyl)-2-phenyl-5-(4-pyridyl)-1H-imidazole was found to be effective in inhibiting B-Raf mediated phosphorylation of GST-kdMEK substrate (IC₅₀ 24nm).

The activity of compounds as Raf inhibitors may also be determined by the assays described in WO 99/10325, McDonald, O.B., Chen, W.J., Ellis, B., Hoffman, C., Overton, L., Rink, M., Smith, A., Marshall, C.J. and Wood, E.R. (1999) "A scintillation proximity assay for the Raf/MEK/ERK kinase cascade: high throughput screening and identification of selective enzyme inhibitors.", *Anal. Biochem.*, 268: 318-329 and AACR meeting New Orleans 1998 Poster 3793.

The neuroprotective properties of Raf inhibitors may be determined by the following *in vitro* assay:

10 Neuroprotective properties of 4-(4-Chloro-3-hydroxyphenyl)-2-phenyl-5-(4-pyridyl)-1H-imidazole in rat hippocampal slice cultures

Organotypic cultures provide an intermediate between dissociated neuronal cell cultures and *in-vivo* models of oxygen and glucose deprivation (OGD). The majority of glial-neuronal interactions and neuronal circuitry are maintained in cultured hippocampal slices, so facilitating investigation of the patterns of death among differing cell types in a model that resembles the *in vivo* situation. These cultures allow the study of delayed cellular damage and death 24 hours, or more, post-insult and permit assessment of the consequences of long-term alterations in culture conditions. A number of laboratories have reported delayed neuronal damage in response to OGD in organotypic cultures of the hippocampus (Vornov *et al*, *Stroke*, (1994) 25: 457-465, Newell *et al*, *Brain Res.*, (1995a) 676: 38-44). Several classes of compounds have been shown to protect in this model, including EAA antagonists (Strasser *et al*, *Brain Res.*, (1995) 687: 167-174), Na channel blockers (Tasker *et al*, *J Neurosci.*, (1992) 12: 4298-4308) and Ca channel blockers (Pringle *et al*, *Stroke*, (1996) 27: 2124-2130). To date, relatively little is known of the roles of intracellular kinase mediated signalling pathways in neuronal cell death in this model.

Method: Organotypic hippocampal slice cultures were prepared using the method of Stoppini *et al*, *J. Neurosci. Methods.*, (1991) 37, 173-182. Briefly, 400 micron sections prepared from hippocampi of 7-8 day postnatal Sprague Dawley rats are cultured on semiporous membranes for 9-12 days. OGD is then induced by incubation in serum and glucose-free medium in an anaerobic chamber for 45 minutes. Cultures are then returned to the air / CO₂ incubator for 23 hours before analysis. Propidium iodide (PI) is used as an indicator of cell death. PI is non toxic to neurones and has been used in many studies to ascertain cell viability. In damaged neurons PI enters and binds to nucleic acids. Bound PI shows increased emission at 635nm when excited at 540nm. One PI fluorescence image and one white light image are taken and the proportion of cell death analysed. The area of region CA1 is defined from the white light image and superimposed over the PI image. The PI signal is thresholded and area of PI damage expressed as a percentage of the CA1 area. Correlation between PI fluorescence and histologically

confirmed cell death has been validated previously by Nissl-staining using cresyl fast violet (Newell *et al*, *J Neurosci.*, (1995b) 15: 7702-7711).

Results: 4-(4-Chloro-3-hydroxyphenyl)-2-phenyl-5-(4-pyridyl)-1*H*-imidazole showed significant protection when pre-incubated 1 hour before a 45 minute OGD

5 (Figure 1). The IC₅₀ for this protection was approximately 100nM.

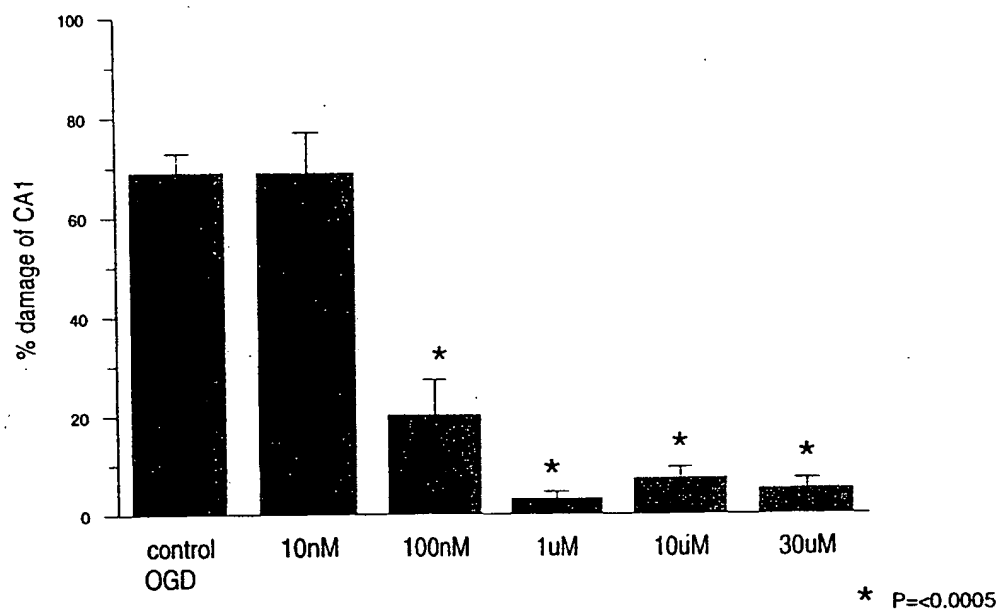
Figure 1: Effect of 4-(4-chloro-3-hydroxyphenyl)-2-phenyl-5-(4-pyridyl)-1*H*-imidazole on oxygen-glucose deprivation-induced neuronal cell death in organotypic hippocampal culture. Duration of OGD 45 minutes. The graph shows the percentage of the neuronal CA1 area that is dead as defined by PI staining. Control values are between 65-75 percent. Results are shown as mean \pm SEM *** (P=<0.0005) n= 6-12.

10 **Conclusions:** B-Raf inhibitors are effective inhibitor of the catalytic activity of human B-Raf protein kinase towards its physiologic substrate MAP kinase (MEK). Moreover, B-Raf inhibitors are potent inhibitors of neuronal cell death that results from oxygen glucose deprivation in hippocampal slice cultures. Such

15 neuroprotective properties indicate that B-Raf inhibitors are likely to be of value in prevention of neuronal cell death associated with ischemic stroke and trauma injury.

Claims:

1. A method of treatment or prophylaxis of a neurotraumatic disease, in a mammal
5 in need thereof, which comprises administering to said mammal an effective amount of a
Raf inhibitor.
2. The use of a Raf inhibitor in the manufacture of a medicament for the
prophylactic or therapeutic treatment of any disease state in a human, or other mammal,
10 which is exacerbated or caused by a neurotraumatic event.
3. The method or use according to claim 1 or 2 wherein the Raf inhibitor is a small
molecule Raf inhibitor.
- 15 4. The method or use according to any one of the preceding claims wherein the Raf
inhibitor is a B-Raf inhibitor.
5. The method or use according to claim 4 wherein the B-Raf inhibitor is 4-(4-
chloro-3-hydroxyphenyl)-2-phenyl-5-(4-pyridyl)-1*H*-imidazole.
20
6. The method or use according to any one of the preceding claims wherein the
neurotraumatic disease is ischemic stroke.
7. The method or use according to any one of the preceding claims wherein the
25 neurotraumatic disease is caused by surgery, or is an open head injury.
8. The method or use according to any one of the preceding claims wherein the
neurotraumatic disease is a closed head injury.

FIGURE 1